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Progress Report on Grant N00014-91-J-1217

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GRANT TITLE: In Vivo Distribution of Liposome Encapsulated Hemoglobin Studied With Imaging Radiotracers

START DATE: 12/01/90

RESEARCH OBJECTIVE: This project has as its objective the development of radiotracer imaging technology to follow the in vivo circulation and organ deposition of liposome encapsulated hemoglobin (LEH). LEH will be labeled with technetium-99m (^{99m}Tc) or indium-111 (^{111}In) and infused into small animals to monitor any in vivo differences between different LEH formulations. These studies will be correlated with any hematological and pathological changes associated with LEH treatment. Development of such non-invasive monitoring techniques may lead to significant cost effective manufacturing and formulation improvements, and ultimately a more efficacious LEH product. The development of this elegant labeling technique should make it possible to study the effect of various LEH modifications on biodistribution non-invasively in primates and humans.

PROGRESS: Our research progress for the period of August 1, 1993 to December 1, 1993 is covered in this report. We have conducted experiments to determine the efficacy of LEH using a positron emitting isotope of oxygen (^{15}O). To our knowledge, this study is the first attempt to actually quantitate and determine oxygen delivery to tissues. These studies are uniquely possible at our institution because of our newly operational cyclotron and positron emission tomography (PET) camera located at our Research Imaging Center and the previous experience of our group in both imaging and blood substitutes. The biodistribution studies using ^{99m}Tc have been very useful, but do not provide any information about how efficient LEH is in delivering oxygen to the tissues in vivo. In initial experiments, we attempted to quantitatively determine the

relative uptake of ^{15}O-O_2 from the lungs by LEH. The percent uptake by LEH was compared to the uptake of the remaining red blood cells following a 40% hypovolemic exchange. To provide an access for blood withdrawal, rats were catheterized via their femoral artery two days prior to the experiments. On the day of the experiment, the rats were anesthetized with pentobarbital and intubated with a converted 14 gauge intravenous catheter, to provide an access to the lungs for delivery of the ^{15}O-O_2. After a short stabilization period, the animals were bled at 1 ml/min and infused with LEH or free hemoglobin at 1 ml/min. At 15 minutes, 3 hours, and 24 hours after LEH or hemoglobin administration, the rats were given a bolus of ^{15}O-O_2. Since the half-life of ^{15}O is approximately 2 minutes, multiple time points can be obtained in the same rat. Blood samples were taken in capillary tubes over a two minute period, and separated into plasma, LEH, and red blood cell fractions by centrifugation. Each fraction was counted in a well counter. Following decay correction, the counts for each fraction were compared as a percentage of the total counts at a particular time point. These experiments showed that LEH and free hemoglobin could load and unload oxygen in the lungs. Also oxygen was removed from the LEH at a rate similar to oxygen removal from the red blood cells. Our initial research shows that this technique for quantitatively determining oxygen uptake and delivery is feasible and has many applications for the development of blood substitutes. This technique applies not only to LEH, but can be used with other oxygen carriers such as cross-linked hemoglobin. This technique allows direct comparisons of various oxygen carriers over an extended time period. We envision that the technique will become an important quality control test for individual batches of oxygen carriers. This technique could also prove invaluable for the development of improved blood substitutes.

We received a shipment of LEH containing human hemolysate from the Naval Research Laboratory for ^{15}O-O_2 efficacy studies. This hemolysate contains methemoglobin reductase, which is the enzyme responsible for converting methemoglobin to oxyhemoglobin. Unfortunately, this batch of LEH was frozen upon arrival. Following centrifugation of the thawed material, we noticed that the supernatant contained approximately 23% of the hemolysate, and therefore determined the LEH was not suitable for these studies. We did test the LEH for methemoglobin levels and they remained low (4.25%) even after freezing. We also determined that this formulation could be labeled with ^{99m}Tc (approximately 40% labeling efficiency), although no extra glutathione was included in the preparation. We injected the LEH (30% topload) in 2

rats and measured the clearance kinetics by blood sampling. There was approximately 50% of the LEH remaining in circulation at 24 hours post-injection. The human hemolysate containing LEH remained red for a longer period of time in vivo than the current LEH formulation made with bovine hemoglobin. This new LEH formulation seems promising and warrants further testing, because one problem we noticed in our previous ^{15}O-O_2 studies with the bovine hemoglobin formulation is that there is a decrease in oxygen carrying capacity by 24 hours post-injection.

We completed a feasibility study to determine if ^{99m}Tc liposomes can be used as blood pool imaging agents in gated cardiac studies and gastric bleeding studies. Today, the most common blood pool agent is autologous red cells that have been labeled with ^{99m}Tc and then reinjected. Normally, these labeled red cells at 24 hours show a urine excretion rate of 40%. Development of ^{99m}Tc liposomes as a blood pool agent, which is more stable in vivo to allow for longer or repeated imaging and does not require handling blood products, would be advantageous. We have developed a method to produce liposomes coated with polyethylene glycol (PEG) and encapsulating glutathione for labeling with ^{99m}Tc, and compared this labeled liposome formulation with labeled red blood cells.

We began two pilot studies to determine if ^{99m}Tc liposomes can be used to image inflammation and infection sites in the abdomen. These models are more clinically relevant than the previous focal infection imaging study in rats. The localization of abdominal sites using ^{99m}Tc liposomes shows promise due to the excellent stability of the ^{99m}Tc label in vivo with little accumulation of ^{99m}Tc in the bowel. One model is appendicitis in rabbits. In this model, the appendix is surgically obstructed which leads to inflammation and migration of white cells to the region. At various times following the initial surgery, an injection of ^{99m}Tc liposomes was given. The animals were imaged under the gamma camera at various times post-injection to determine if the appendix could be located. After 24 hours, an autopsy was performed to inspect the location of the appendix in relation to the images. Samples of tissue were taken and counted in a well counter. Only later stage appendicitis was detectable by this imaging technique. The second model was localization of a focal abdominal infection in rabbits. Focal abdominal abscesses are difficult to treat with antibiotics and normally require surgical drainage. The development of an accurate and practical imaging technique would help the surgeon locate the area for drainage. In this model, ping pong balls containing 250 holes were implanted in the abdomen of rabbits.

Sixty days later after the balls were filled with fluid and connective tissue, they were innoculated percutaneously with bacteria to form an infection. Twenty-four hours later, the animals were injected with ^{99m}Tc liposomes. Gamma camera images were acquired. The current model could not sufficiently localize the infected ball from the uninfected ball. The location of the balls' surface could be located, but the balls did not accumulate the ^{99m}Tc liposomes, producing a hot spot easily visualized in the gamma camera images. Upon examination at autopsy, the balls were coated with a fibrous material and the liposomes could not penetrate the ball to localize the infection. We feel this second model probably does not mimic the clinical situation and another model must be developed.

WORK PLAN: During the next funding period, we will continue to use our ^{99m}Tc liposome labeling protocol to test LEH formulations as supplied by NRL or Vestar for their circulation properties and organ distribution. A LEH formulation being developed by Vestar which can be produced at a smaller more homogeneous size for sterile filtration has been modified for scale up. Biodistribution studies with these new LEH preparations will be studied as soon as these preparations become available.

We plan to continue our ^{15}O-O_2 studies to determine the efficacy of different formulations of LEH. Dr. Alan Rudolph will be sending us lyophilized LEH samples to test for its ability to load and unload oxygen. We plan to perform similar experiments as outlined in the progress section of this report. These studies will provide important information concerning this storage form of LEH. We also plan to study LEH containing human hemolysate to determine if this formulation will carry oxygen for a longer period of time than the current formulation.

Although our ^{99m}Tc labeling procedure has provided valuable data concerning the biodistribution of LEH, it does not allow us to follow the ultimate metabolic fate of the hemoglobin. This information is very important for the safety of LEH as a blood substitute since we want a product which will be cleared from the body and produce few toxic side effects. To study this problem, we plan to label hemoglobin with ^{3}H and ^{14}C using a mild reductive methylation procedure. This mild labeling technique has been used to label the lysine residues of a number of proteins including hemoglobin without affecting the functionality of the protein. The hemoglobin will be supplied by NRL. Also the radiolabeled starting material used in the procedure is available from commercial sources. Once labeled, the hemoglobin will be used to make LEH. The

labeled LEH will then be double labeled using the ^{99m}Tc liposome labeling protocol. This double labeled material will be injected into animals and imaged under the gamma camera. The animals will then be sacrificed for tissue biodistribution measurements. Samples of the tissues will be counted for both gamma activity as well as for ^{3}H or ^{14}C using liquid scintillation counting. This study will provide important information concerning the fate of both the hemoglobin and liposomal components of LEH.

INVENTIONS: A licensing agreement between Lipotek INC and The University of Texas Health Science Center at San Antonio/Department of the Navy has been approved.

PUBLICATIONS AND REPORTS: The article entitled " Biodistribution and Imaging Studies of Technetium-99m-Labeled Liposomes in Rats with Focal Infection" was published in the December issue of the Journal of Nuclear Medicine. A poster entitled "Use of a New ^{99m}Tc Liposome Labeling Method for Diagnostic Imaging and Drug Targeting Applications" was presented at the Liposomes In Drug Delivery: The Nineties and Beyond meeting held in London, England on December 13-17, 1993. We are currently writing manuscripts describing our platelet studies, ^{15}O-O_2 studies, and the biodistribution of ^{99m}Tc labeled LEH in a rat hypovolemic model.

TRAINING ACTIVITIES: None